

# Adjuvant L-arginine treatment in controlled ovarian hyperstimulation: a double-blind, randomized study

Cesare Battaglia<sup>1,4</sup>, Giorgia Regnani<sup>3</sup>, Tiziana Marsella<sup>3</sup>, Fabio Facchinetti<sup>3</sup>, Annibale Volpe<sup>3</sup>, Stefano Venturoli<sup>1</sup> and Carlo Flamigni<sup>2</sup>

<sup>1</sup> Reproductive Medicine Unit and <sup>2</sup> Department of Obstetrics and Gynaecology, University of Bologna and <sup>3</sup> Department of Obstetrics and Gynaecology, University of Modena, Italy

## Abstract

**BACKGROUND:** Enhanced vascularization appears to be important for follicular selection and maturation in both spontaneous and stimulated IVF cycles. Nitric oxide, formed *in vivo* from L-arginine, may play a key role in follicular maturation and ovulation. **METHODS:** To evaluate the role of L-arginine supplementation in controlled ovarian hyperstimulation, 37 IVF patients were divided into two groups according to ovarian stimulation protocols: group I, GnRH agonist plus pure (p)FSH plus oral L-arginine ( $n = 18$ ); and group II, GnRH agonist plus pFSH plus placebo ( $n = 19$ ). Hormonal, ultrasonographic and Doppler evaluations were performed, and plasma and follicular fluid nitrite/nitrate concentrations were monitored. **RESULTS:** Thirty-two patients completed the study. In group I ( $n = 16$ ), plasma L-arginine concentrations increased from (basal)  $87 \pm 12 \mu\text{mol}$  to  $279 \pm 31 \mu\text{mol}$  ( $P = 0.002$ ) on the day of  $\beta$ -HCG administration. In this group, pFSH treatment was shorter ( $P = 0.039$ ) than in group II ( $n = 16$ ). The number of the follicles 17 mm was lower ( $P = 0.038$ ) in group I than group II. The 'good quality' embryos were fewer in number ( $P = 0.034$ ) and pregnancy rate, both per patient ( $P = 0.024$ ) and per embryo transfer ( $P = 0.019$ ), was lower in group I. In the L-arginine group, an increased follicular fluid concentration of nitrite/nitrate was observed. On day 8 of the cycle, elevated plasma estradiol levels were associated with decreased blood flow resistances of perifollicular arteries. Follicular fluid concentrations of nitrite/nitrate were inversely correlated with embryo quality ( $r = -0.613$ ;  $P = 0.005$ ) and perifollicular artery pulsatility index ( $r = -0.609$ ;  $P = 0.021$ ). **CONCLUSIONS:** L-Arginine supplementation may be detrimental to embryo quality and pregnancy rate during controlled ovarian hyperstimulation cycles.

*Key words:* controlled ovarian hyperstimulation/Doppler/IVF/L-arginine/nitric oxide

## Introduction

Helping infertile couples to have healthy children is one of the primary tasks of assisted reproductive technologies. In order to fulfil this task, reproductive medicine constantly needs to obtain information on physiology and pathophysiology of infertility, and to develop efficient strategies for controlled ovarian hyperstimulation.

The regulation and significance of ovarian and uterine haemodynamics in human reproductive pathophysiology is becoming an important research area, and transvaginal colour flow Doppler ultrasound facilitating the detection of small vessels and the measurement of impedance to flow in the utero-ovarian circulation may represent an important tool for studying the female reproductive system and pelvic haemodynamics.

An increased vascularization of ovarian follicles during the course of their development occurs in experimental animals (Koning *et al.*, 1989). In women, an enhanced vascularization seems to be responsible for the selection and maturation of follicles both in spontaneous and stimulated IVF cycles (Weiner *et al.*, 1993; Balakier and Stronell, 1994; Bassil *et al.*, 1997). Gonadotrophins, steroids, prostaglandins and other vasoactive molecules are involved in the regulation of ovarian blood flow (Taymor, 1996). The importance of nitric oxide (NO) as an intra- and intercellular modulator has been

recognized in many biological processes, including ovarian physiology (Anteby *et al.*, 1996). NO is a labile and diffusible molecule which forms stable oxidized metabolites (nitrite/nitrate; NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>) detectable in many biological fluids. *In vivo*, NO is formed from L-arginine either by a constitutive calcium-dependent, or a pro-inflammatory cytokine-inducible, NO synthase (Moncada *et al.*, 1991). Although the precise role of NO has not been elucidated, it has been suggested that it is involved in follicular maturation and ovulation (Anteby *et al.*, 1996; Tao *et al.*, 1997). It has also been suggested that NO may participate in periovulatory vasodilatory modulation of rat ovarian blood flow (Ben-Shlomo *et al.*, 1994).

The role of NO in IVF has been recently evaluated (Manau *et al.*, 2000). These authors showed a lack of relationship between intrafollicular nitrite/nitrate concentrations and ovarian response and IVF outcome (i.e. fertilization and pregnancy rate). However, in a previous paper (Battaglia *et al.*, 1999), it was shown that oral L-arginine supplementation during controlled ovarian hyperstimulation in poor responder patients decreases blood flow resistance in both perifollicular and uterine arteries. Hence it was speculated that L-arginine, by modulating the permeability of follicular epithelium to plasma proteins and increasing uterine perfusion, might improve ovarian response, endometrial receptivity and pregnancy rate.

The aim of the present study was to evaluate, prospectively, blindly and randomly, the possible role of orally administered L-arginine in modifying vascular parameters and improving ovarian response to gonadotrophins in IVF cycles in normally responding women.

## Materials and methods

### Patients and protocols

The study protocol was approved by the Institutional Ethics Review Committee. The patient sample size calculation was computed with regard to the number of follicles >17 mm maximum diameter. This parameter was considered the primary outcome. It was calculated that to obtain an arbitrarily chosen potential difference of 1.7 follicles of >17 mm diameter among treated and untreated women, a sample size of 15 patients would provide 90% power at a significance level of 0.05. All 37 women attending the Modena University Infertility Clinic who participated the study provided their informed consent.

The mean ( $\pm$  SD) age of the patients was 33.8  $\pm$  3.1 years (range 28–37), and the mean duration of infertility was 3.7  $\pm$  2.4 years (range 2–6). All patients were selected from women who suffered from tubal infertility. All had regular menstrual cycles (28  $\pm$  4 days), and their partners were fertile according to World Health Organization standards. Patients with concurrent illness were excluded from the study. Other exclusion criteria included body mass index [BMI = weight (kg)/height (m)<sup>2</sup>]  $\geq$  30, endometriosis, ovarian functional cyst, polycystic ovarian syndrome, unilateral ovarian resection or ovariectomy. Likewise, patients who took regular exercise, were heavy smokers (>10 cigarettes/day), and were hypertensive (systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg) were excluded from the study. None of the women had received hormonal treatments for at least 4 months before the IVF attempt.

In order to assess ovarian reserve, peripheral blood was obtained from all patients between 08:00 and 11:00 on day 3 of the cycle preceding the IVF attempt, after an overnight fast. Basal plasma estradiol (E<sub>2</sub>), FSH and LH concentrations were determined using a radioimmunoassay (RIA; Radim, Pomezia, Italy).

Patients were assigned randomly to two different stimulation protocols: a long GnRH agonist protocol and pure (p)FSH plus oral L-arginine (group I; *n* = 18); or a long GnRH agonist protocol and pFSH plus placebo (group II; *n* = 19). The placebo resembled (in terms of ampoule appearance, smell and flavour) the corresponding L-arginine preparation, and both the participating women and investigators were unaware which treatment was received. Randomization was performed by opening sequentially numbered sealed envelopes containing treatment allocation determined by a random number table.

Controlled ovarian hyperstimulation was achieved by an i.m. injection on day 20 of the cycle of GnRH agonist triptorelin (Decapeptyl 3.75; Ipsen, Milan, Italy) and, after pituitary desensitization (plasma E<sub>2</sub> concentration <100 pmol/l; ovaries with no follicles >5 mm in diameter and endometrial thickness <5 mm), i.m. administration of pFSH (Metrodin 75 HP; Serono, Rome, Italy; 225 IU in the first 3 days of the cycle, then in an individually assessed dosage).

Patients were supplemented with 2x4 ampoules/day of either oral L-arginine (Bioarginina; Damor, Napoli, Italy; one ampoule = 2 g L-arginine) or placebo.

The IVF cycles were cancelled when E<sub>2</sub> plasma levels were <1.1 pmol/l and/or fewer than three follicles were recruited by cycle day 8. Similarly, the IVF cycles were cancelled in those patients at risk of ovarian hyperstimulation syndrome (= " src="/math/ge.gif" border=015 follicles per ovary and/or plasma E<sub>2</sub> levels = " src="/math/ge.gif" border=09000 pmol/l).

When at least two follicle >17 mm in diameter were present, triptorelin, pFSH and L-arginine or placebo were withdrawn and 10 000 IU HCG (Profasi; Serono) were administered i.m. Ultrasonographic oocyte recovery was performed transvaginally 35–36 h after HCG injection. The retrieved oocytes were classified as mature, immature or atretic on the basis of the morphology and appearance of the oocyte cumulus–corona complex according to published criteria (Acosta *et al.*, 1984). In order to study the impact of embryo quality on implantation, the embryos were graded morphologically before replacement. The embryos were scored as follows: grade A, equal-sized blastomeres, no fragmentation; grade B, equal- or unequal-sized blastomeres, <20% fragmentation; grade C, equal- or unequal-sized blastomeres, 20–50% fragmentation; and grade D, equal- or unequal-sized blastomeres, >50% fragmentation. Embryo transfer was performed 72 h after oocyte retrieval. Between one and three embryos were replaced at the 6- to 12-cell stage. Transcervical transfer was carried out using a Frydman catheter (SCS International, Genoa, Italy). The remaining cleaved embryos with <20% fragmentation were allocated to a cryopreservation protocol. Vaginal progesterone (Esolut; Angelini, Rome, Italy) was prescribed as luteal phase support until the serum β-HCG assay was performed. A clinical pregnancy was diagnosed by ultrasonographic evidence of embryonic heart activity.

During the ovarian stimulation regimen the patients were submitted to hormonal (E<sub>2</sub>), biochemical (L-arginine) and ultrasonographic (follicular number and diameter, endometrial thickness) and Doppler (uterine and perifollicular arteries) evaluations. Plasma and follicular fluid concentrations of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> were assayed.

### **Ultrasound and Doppler examinations**

Transvaginal ultrasonographic assessments of endometrial thickness were performed on days 1 and 8 of ovarian stimulation, and on the day of HCG administration in both groups, using a 6.5 MHz vaginal transducer (A4 Idea; Esaote, Milan, Italy). Measurements of follicular size were performed daily beginning on day 8 of the cycle until the day of oocyte retrieval. A modified ovarian synchrony index (OSI = number of follicles >17 mm/number of follicles 10–14mmx100) (Franco *et al.*, 1994) was calculated.

Doppler flow measurements of uterine and perifollicular arteries were performed transvaginally with a 6.5 MHz (A4 Idea) colour Doppler system. The Doppler examination was performed at the beginning of pFSH administration, on day 8 of controlled ovarian hyperstimulation and on the day of oocyte retrieval. All patients were studied between 08:00 and 11:00 in order to exclude the effects of circadian rhythm on blood flow (Zaidi *et al.*, 1995b). Patients were allowed to rest for at least 15 min before being scanned, and completely emptied their bladder in order to minimize any external effects on blood flow (Battaglia *et al.*, 1994). A 50 Hz filter was used to eliminate low-frequency signals originating from vessel wall movements. The maximum ultrasonographic energy was <80 mW/cm<sup>2</sup>. The intensity was within the safety limits suggested by the American Institute for Ultrasound in Medicine (Lizzi and Mortimer, 1989). Colour

flow images of the ascending branches of the uterine arteries were sampled lateral to the cervix in a longitudinal plane. The angle of insonation was altered to obtain the maximum colour intensity. When good colour signals were obtained, blood flow velocity waveforms were recorded by placing the sample volume across the vessel and entering the pulsed Doppler mode. The pulsatility index (PI), defined as the difference between peak systolic (S) and end-diastolic (D) flow velocity divided by the mean flow velocity ( $S - D / \text{mean}$ ) was calculated electronically. The PI has been shown to reflect blood flow impedance, and may be used when the end-diastolic frequency shift is absent or reversed. For each examination the mean value of three consecutive waveforms was obtained. No significant differences between the PI of the left and right uterine arteries were observed, and hence the average value of both arteries was used. The perfollicular arteries, starting from day 8 of controlled ovarian hyperstimulation, were identified around the follicles ( $>1.0$  cm maximum diameter), in the ovarian stroma at the maximum distance from the surface of the ovary. Recorded spectra were analysed and the resistance index (RI) was obtained ( $RI = S - D / S$ ). Arteries demonstrating the lowest downstream impedance were selected for measurements, assuming that these were the branches supplying the developing follicles directly. When calculating results, the PIs of both uterine and perfollicular arteries were not corrected for heart rate. An indication of within-patient precision of the Doppler procedures was obtained by analysing the flow velocity waveforms recorded on three occasions either from uterine and perfollicular arteries at 1 min intervals. An analysis of variance of the results from 15 patients gave a mean coefficient of variation of 5.3% for uterine and 6.7% for perfollicular arteries, and showed no significant differences between the replicate analyses. Ultrasonographic and Doppler analyses were performed by one examiner (C.B.).

### **Hormonal and biochemical assays**

Peripheral blood was obtained between 08:00 and 11:00 (after an overnight fast) on day 1, day 8, and on the day of HCG administration. The blood was immediately centrifuged, and the serum removed and stored at  $-70^{\circ}\text{C}$  until taken for assay. Estradiol was measured by RIA as reported above; plasma L-arginine concentrations were assessed as described previously (Facchinetti *et al.*, 1998).

NO production was assessed by monitoring (on day 1, day 8 and day of oocyte retrieval) plasma levels of stable oxidation products of NO metabolism ( $\text{NO}_2^- / \text{NO}_3^-$ ). Since very little or no  $\text{NO}_2^-$  is normally found in the serum, no attempt was made to differentiate between  $\text{NO}_2^-$  and  $\text{NO}_3^-$  amounts; hence, results were reported as  $\text{NO}_2^- / \text{NO}_3^-$ .  $\text{NO}_2^- / \text{NO}_3^-$  were assayed using the Greiss reaction with previously described procedures (Clancy and Abramson, 1992; Facchinetti *et al.*, 1997).

$\text{NO}_2^- / \text{NO}_3^-$  levels were also assayed in follicular fluid in those patients who reached oocyte retrieval. Following transvaginal needle aspiration of the accessible follicles, in order to homogenize the fluids and to reduce possible interfollicular differences, the follicular fluids of follicles were pooled and immediately centrifuged (2000 g for 20 min). The supernatant was removed and stored at  $-70^{\circ}\text{C}$  until bioassayed. Similarly, aliquots of follicular fluid obtained by aspiration of all accessible follicles ( $<17$  mm) were pooled, centrifuged, stored at  $-70^{\circ}\text{C}$ , and subsequently assayed. The analyses were performed using the same methods as for serum assays.

All samples from each subject were analysed in duplicate in the same assay. On the basis of two quality control samples, the average intra- and inter-assay coefficients of variation were 5.1 and 7.7% for LH, 4.8 and 7.1% for FSH, 4.9 and 7.5% for  $\text{E}_2$ , and 6.8 and 11.3% for L-arginine respectively. In addition the  $\text{NO}_2^- / \text{NO}_3^-$  intra- and inter-assay coefficients of variation were 6.6 and 8.9% respectively. No differences were observed between follicular fluid and serum assays.

### **Statistical analysis**

A statistical analysis was performed using the Mann–Whitney,  $\chi^2$ , Fisher–Irwin exact and Wilcoxon tests and a one-way analysis of variance, where indicated. The relationship between the parameters analysed was assessed using the linear regression method. A *P*-value 0.05 was considered to be statistically significant.

Data were presented as mean  $\pm$  SD, unless otherwise indicated.

## Results

Hormonal evaluation on day 3 of the cycle preceding the IVF attempt confirmed normal ovarian reserves in both patient groups (Table I)

**Table I.** Ovarian reserve on day 3 of the cycle preceding the IVF attempt in L-arginine- and placebo-treated groups<sup>a</sup>

Hormone	L-Arginine <sup>b</sup> ( <i>n</i> = 16)	Placebo <sup>b</sup> ( <i>n</i> = 16)	Normal range <sup>c</sup>
FSH (IU/l)	5.9 $\pm$ 1.5	5.9 $\pm$ 1.9	1.5–10
LH (IU/l)	4.1 $\pm$ 1.8	4.7 $\pm$ 1.0	1.5–10
Estradiol (pmol/l)	146 $\pm$ 51	138 $\pm$ 64	<180
<sup>a</sup> No significant differences were observed between the groups.			
<sup>b</sup> Values are mean $\pm$ SD.			
<sup>c</sup> Normal range derived from normally ovulating patients attending the infertility clinic.			

Thirty-two patients completed the study. The cancellation rate, due to a poor response, was 2/18 (11.1%) and 3/19 (15.7%) in groups I and II respectively. No significant side-effects were reported by patients in either the L-arginine- or placebo-treated groups. The number of pFSH ampoules and pFSH units/day did not differ significantly between groups (Table II). However, the duration of pFSH treatment was significantly longer in the placebo-treated group (12.3  $\pm$  3.5 days) than the L-arginine group (10.6  $\pm$  2.4 days; *P* = 0.039). The number of recruited follicles on the day of HCG administration was higher in group I (15.3  $\pm$  3.3) than in group II (10.4  $\pm$  3.1; *P* = 0.021). The number of large ( $\geq$  17 mm maximum diameter) and small (10–14 mm maximum diameter) follicles, as well as the OSI (an index of follicular growth homogeneity) and endometrial thickness on the day of HCG administration are reported in Table II. Although, between groups, the number of large ( $\geq$  17 mm) and small (<14 mm) follicles reached only a weak significant difference, the OSI was significantly higher in placebo-treated patients (48.5  $\pm$  12.8%) than in those receiving L-arginine (32.3  $\pm$  11.6%; *P* = 0.004).

**Table II.** Response to controlled ovarian hyperstimulation in the L-arginine- and placebo-treated groups

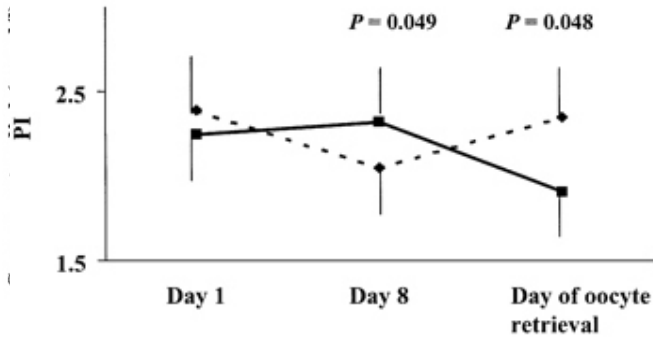
Parameter	L-Arginine <sup>a</sup> (n = 16)	Placebo <sup>a</sup> (n = 16)	P-value
No. of pFSH ampoules	31.5 ± 10.2	37.6 ± 6.1	NS
No. of days of pFSH treatment	10.6 ± 2.4	12.3 ± 3.5	0.039
pFSH units/day	3.2 ± 0.8	2.5 ± 0.9	NS
No. of recruited follicles	15.3 ± 3.3	10.4 ± 3.1	0.021
Follicles = " src="/math/ge.gif" border=017 mm	4.3 ± 2.1	6.1 ± 2.1	0.038
Follicles 14–16 mm	3.7 ± 1.8	2.2 ± 1.1	0.057
Follicles 10–14 mm	7.4 ± 1.7	3.3 ± 2.0	0.034
Ovarian synchrony index (%)	32.3 ± 11.6	48.5 ± 12.8	0.004
Endometrial thickness (cm)	1.07 ± 0.11	1.09 ± 0.17	NS
<sup>a</sup> Values are mean ± SD.			
NS = not significant.			

The number and quality of oocytes collected, and the fertilization rate (number of oocytes fertilized/number of oocytes collected x100) did not differ significantly between the two groups (Table III). However, embryo morphology (an indirect expression of embryo quality) was significantly better in group II (grade A + grade B = 72.1 ± 15.6%) than in group I (grade A + grade B = 50.0 ± 26.3%; *P* = 0.034). The number of embryos transferred was similar in both groups (2.6 ± 0.5 versus 2.8 ± 0.3). The pregnancy rate per cycle (16.6 versus 31.6%; *P* = 0.024) and pregnancy rate per embryo transfer (18.7 versus 37.5%; *P* = 0.019) was significantly higher in the placebo- than the L-arginine-treated group. Among the nine pregnancies obtained, seven resulted in live births and two are currently near-term ongoing pregnancies.

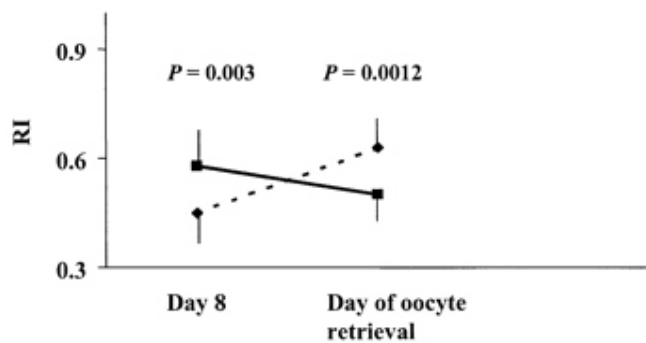
**Table III.** Response to controlled ovarian hyperstimulation in the L-arginine- and placebo-treated groups

Parameter	L-Arginine <sup>a</sup> (n = 16)	Placebo <sup>a</sup> (n = 16)	P-value
No. of oocytes collected	9.9 ± 3.4	9.2 ± 2.8	NS
Oocyte morphology (%)			
Mature	64.2 ± 15.7	78.5 ± 24.6	NS
Immature	32.5 ± 13.4	21.4 ± 24.6	NS
Atretic	6.5 ± 6.5	0	
Fertilization rate (%)	68.4 ± 23.7	71.3 ± 22.9	NS
Embryo morphology (%)			
Grade A + Grade B	50.0 ± 26.3	72.1 ± 15.6	0.034
Grade C + Grade D	49.9 ± 36.1	27.3 ± 14.7	NS
<sup>a</sup> Values are mean ± SD.			
NS = not significant.			

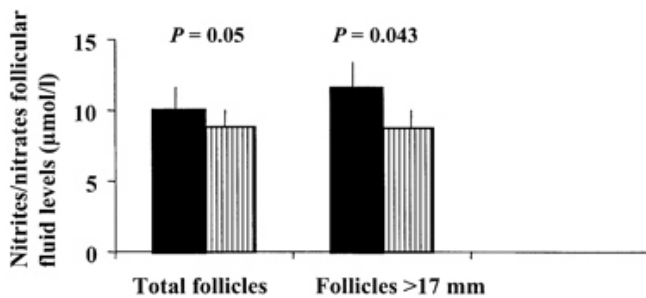
During ovarian stimulation, serum E<sub>2</sub> levels were increased (Figure 1). The plasma concentration of L-arginine was increased in the treated group, from 87 ± 12 (basal) to 279 ± 31 μmol/l (day of HCG administration) (*P* = 0.002). No significant differences were observed between basal and HCG day plasma L-arginine levels in the placebo group (68 ± 11 versus 56 ± 19 μmol/l). Plasma NO<sub>2</sub>-/NO<sub>3</sub>- levels were also increased during ovarian stimulation (Figure 2). In the follicular fluid, NO<sub>2</sub>-/NO<sub>3</sub>- concentrations were higher in the L-arginine (9.87 ± 1.35 μmol/l) than placebo (8.68 ± 1.82 μmol/l; *P* = 0.048) -treated group, the difference becoming more evident (11.56 ± 3.42 versus 8.75 ± 1.51 μmol/l; *P* = 0.033) when considering only the NO<sub>2</sub>-/NO<sub>3</sub>- concentration in follicles with maximum diameter >17 mm (Figure 2). Uterine and perfollicular blood flow resistances were also changed during ovarian stimulation (Figure 3).



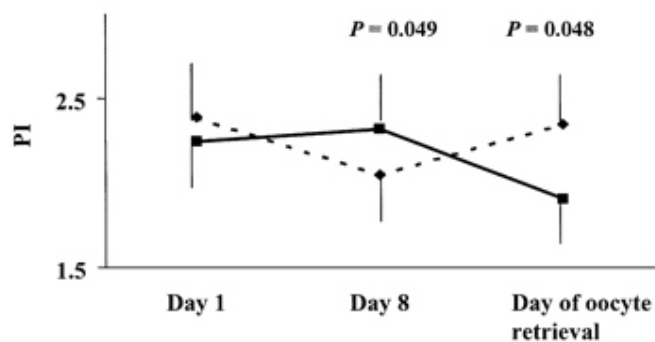
**Figure 1.** Serum estradiol concentrations during ovarian stimulation in L-arginine- (broken line) and placebo- (solid line) treated groups. On day 8 of controlled ovarian hyperstimulation, plasma estradiol levels were significantly higher in the L-arginine-supplemented group.



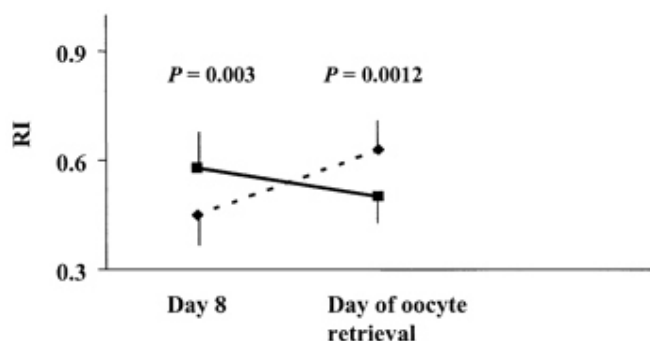
**Figure 2.** Plasma and follicular fluid nitrite/nitrate concentrations. Upper: Plasma levels were significantly higher on day 8 of controlled ovarian hyperstimulation in L-arginine- (broken line) than placebo- (solid line) treated groups. Likewise, in L-arginine-treated women, on day 8 of ovarian stimulation, plasma nitrite/nitrate concentrations were significantly higher than basal concentrations ( $P = 0.002$ ) and on the day of oocyte retrieval ( $P = 0.001$ ).



Lower: Follicular fluid nitrite/nitrate concentrations were significantly higher in L-arginine- (solid box) than placebo- (shaded box) supplemented patients.



**Figure 3.** Uterine artery (upper diagram) and perifollicular artery (lower diagram) blood flow resistances during controlled ovarian hyperstimulation. Significant differences were detectable from day 8 and later between the L-arginine- (broken line) and placebo- (solid line) treated groups. RI = resistance index; PI = pulsatility index.



Among the study population, follicular fluid  $\text{NO}_2^-/\text{NO}_3^-$  concentrations were inversely correlated with embryo quality ( $r = -0.613$ ;  $P = 0.005$ ) and perifollicular artery PI ( $r = -0.609$ ;  $P = 0.021$ ). Furthermore, plasma  $\text{NO}_2^-/\text{NO}_3^-$  concentrations were inversely correlated with uterine artery PI ( $r = -0.476$ ;  $P = 0.046$ ).

## Discussion

In natural cycles, only one follicle is dominant and reaches a mean size of  $>17$  mm maximum diameter, while the remaining follicles attain a maximum diameter  $<10$  mm (Hackeloer *et al.*, 1979

Thus, whilst in the natural cycle the ideal OSI is 100%, in stimulated cycles many follicles are dominant and it is not possible to obtain an ideal OSI. However, a progressive increase of OSI may reflect an increasing homogeneity of follicular cohort. The use of L-arginine resulted in quick, intense and incongruent follicular growth, as indicated by the OSI. It has previously been shown (Eldar-Geva *et al.*, 1998) that incongruent follicular development during controlled ovarian hyperstimulation may have a negative influence on the outcome of gamete intra-Fallopian transfer cycles. Moreover, because it occurred in older patients who were submitted to higher doses of FSH and presented lower serum  $\text{E}_2$  concentrations and fewer retrieved oocytes, the authors speculated that incongruent follicular distribution might be considered an expression of poor follicular development during controlled ovarian hyperstimulation. The results of the present study do not appear to confirm the findings of Eldar-Geva *et al.* as the doses of pFSH, the plasma  $\text{E}_2$  levels on the day of HCG, and the number and quality of oocytes collected did not differ between the two groups. In contrast, because in the L-arginine-treated group (on day 8 of the stimulated cycle) elevated plasma  $\text{E}_2$  levels were associated with high plasma  $\text{NO}_2^-/\text{NO}_3^-$  levels and also with decreased blood flow resistances of perifollicular arteries, it might be speculated that NO derivatives act as vasodilators that cause too early an increase in the permeability of the follicular epithelium to plasma proteins, and that this results in the follicles being susceptible to circulating FSH and growth hormone

(GH) action. The simultaneous early action of FSH and GH may promote a wide and incongruent follicular recruitment with consequent early and not synchronous increased production of insulin-like growth factor-I, which most likely favours a non-homogeneous follicular maturation and differentiation of granulosa cells by modifying various physiological mechanisms (Erikson *et al.*, 1989; Adashi *et al.*, 1991; Artini *et al.*, 1994).

In the present study, although the fertilization rate and the number of transferred embryos were similar in both groups, the quality (type A+B) of embryos and pregnancy rate were higher in placebo-treated patients. Furthermore, in L-arginine-treated women, the intrafollicular NO<sub>2</sub>-/NO<sub>3</sub>- concentrations were higher than in the placebo-supplemented group, whilst in the entire study population the follicular fluid NO<sub>2</sub>-/NO<sub>3</sub>- concentrations were inversely correlated with embryo quality.

Each embryo has its own developmental potential, and few cleaved embryos are competent to implant after IVF and develop through gestation (Van Blerkom *et al.*, 1997). It is known that mature oocytes often contain chromosomal and cytoplasmic structural defects that prevent the fertilized oocytes from adequate developmental growth. How and when such anomalies intervene are not well understood. Although differences in follicle cell function and follicular fluid biochemistry have been suggested to influence the developmental potential of the human oocyte, no single factor—whether secreted into the circulation or present in the follicular fluid—has been shown to provide definitive prediction of the developmental competence of the oocyte–embryo complex. It has been suggested (Gaulden, 1992) that intrafollicular hypoxia might negatively influence spindle organization and chromosomal segregation in the human oocyte. However, the intra-ovarian regulatory system is composed of various substances including growth factors, cytokines, neuropeptides and vasoregulatory molecules and, among these, NO and its derivatives may serve an important role.

Several studies have demonstrated that elevated NO concentrations can reduce cellular ATP levels by inhibiting the cells' ATP-generating ability (Moncada *et al.*, 1991). This cytostatic and cytotoxic mechanism induces a direct inhibition of mitochondrial respiration and DNA synthesis. Furthermore, elevated NO concentrations react actively with oxygen, yielding strongly oxidizing molecules (nitrogen dioxide and peroxy nitrites) that are potentially more toxic than NO itself (Anggard, 1994).

The above considerations allow us to speculate that L-arginine supplementation with the consequent elevated intrafollicular NO<sub>2</sub>-/NO<sub>3</sub>- concentrations may have detrimental effects on embryo quality and pregnancy rate. This may appear to be in contrast to a previous study, where it was affirmed that in 'poor responder' patients the adjuvant L-arginine supplementation during controlled ovarian hyperstimulation improved follicular growth, oocyte quality, fertilization rate, and arguably also pregnancy rate (Battaglia *et al.*, 1999). However, when comparing the intrafollicular content in normal and 'poor' responders, it was noted that L-arginine-supplemented 'poor responders' showed follicular fluid NO<sub>2</sub>-/NO<sub>3</sub>- concentrations ( $6.7 \pm 1.11 \mu\text{mol/l}$ ) (Battaglia *et al.*, 1999) that were similar to those in placebo-treated patients ( $8.68 \pm 1.82 \mu\text{mol/l}$ ;  $P = 0.064$ ) but significantly lower than in L-arginine-supplemented, normally responding women ( $9.87 \pm 1.35 \mu\text{mol/l}$ ;  $P = 0.022$ ). It was speculated that follicular fluid NO derivatives are most likely necessary for oocyte activation at fertilization and have beneficial effects when produced within physiological limits, but at higher doses they can cause cytostatic and cytotoxic effects and have detrimental consequences on embryo quality, implantation and pregnancy rate.

The pregnancy rate was significantly higher in the placebo-treated than the L-arginine-treated group, and this might be due to better embryo quality and/or improved endometrial receptivity. There are no accepted standard criteria for evaluating endometrial receptivity, although attempts have been made to correlate it with ultrasound parameters (Gonen and Casper, 1990; Khalifa *et al.*, 1992; Coulam *et al.*, 1994; Yaron *et al.*, 1994; Noyes *et al.*, 1995). In those patients who reached oocyte retrieval, similar results were obtained in terms of endometrial texture and thickness, with or without L-arginine. These data confirm that endometrial ultrasonography is not helpful in evaluating endometrial receptivity.

The measurement of impedance to uterine blood flow in IVF cycles has provided an indirect measure of endometrial receptivity (Battaglia *et al.*, 1990; Steer *et al.*, 1992; Bassil *et al.*, 1995; Zaidi *et al.*, 1995a, 1996). In the present study, a significantly lower downstream impedance in uterine arteries resulted, on the day of oocyte retrieval, in placebo-supplemented patients. These data confirm that the decrease in peripheral impedance in the uterine vascular bed, reflected by a low PI, is a consequence of increased blood flow and tissue perfusion, which may improve uterine receptivity (Goswamy *et al.*, 1988; Battaglia *et al.*, 1990, 1997; Steer *et al.*, 1992).

In both groups, an inverse correlation between plasma NO<sub>2</sub>-/NO<sub>3</sub>- concentrations and uterine artery Doppler PI was seen. Furthermore, on the day of oocyte retrieval, a significantly lower uterine artery PI was observed in the placebo-treated women. Hence, it might be suggested that, in accordance with data reported by others (Ramsay *et al.*, 1994, 1995) who found that human uterine blood flow can be increased by the administration of a NO donor drug, the relaxation of vascular smooth muscle of endometrial vessels may be partially mediated by NO and its derivatives. However, a sudden reduction in plasma NO<sub>2</sub>-/NO<sub>3</sub>- concentration, seen after the circulation of large quantities of NO<sub>2</sub>-/NO<sub>3</sub>- for a relatively long period, might induce an intense rebound effect on vascular tone, increase the impedance to flow in the uterine vascular bed, and reduce endometrial receptivity.

The above considerations support the hypothesis that an adequate modulation of endometrial vascularity might improve the implantation and pregnancy rate.

Although further larger randomized studies are necessary to elucidate the factors that influence intra-ovarian regulation of ovarian function, it may be concluded that oral L-arginine supplementation in normally responding patients increases follicular recruitment and reduces the duration of pFSH treatment, but might also have detrimental effects on embryo quality and pregnancy rate.

### **Acknowledgements**

The authors thank Dr Michela Salvatori and the staff of the Modena University Infertility Clinic for their invaluable help and cooperation. They also thank Daniele Radi for expert assistance in laboratory assays, and E.Rossi for help with the statistical analysis.

### **Notes**

<sup>4</sup> To whom correspondence should be addressed at: 1st Department of Obstetrics and Gynaecology–Reproductive Medicine Unit, University of Bologna, Via Massarenti, 13 40138 Bologna, Italy. E-mail: [battagli@med.unibo.it](mailto:battagli@med.unibo.it)

Submitted on February 2, 2001; resubmitted on August 3, 2001

### **References**

- Acosta, A.A., Jones, G.S., Garcia, J.E., Sandow, B., Veeck, L. and Mantzavinos, T. (1984) Correlation of human menopausal gonadotropin/human chorionic gonadotropin stimulation and oocyte quality in an *in vitro* fertilization program. *Fertil. Steril.*, **41**, 196–201. [\[ISI\]](#) [\[Medline\]](#)
- Adashi, E.Y., Resnick, C.E., Hurwitz, A., Ricciarelli, E., Hernandez, E.R., Roberts, C.T., Leroith, D. and Rosenfield, R. (1991) Insulin-like growth factors: the ovarian connection. *Hum. Reprod.*, **6**, 1213–1219. [\[Abstract\]](#)
- Anggard, E. (1994) Nitric oxide: mediator, murderer, and medicine. *Lancet*, **343**, 1199–1206. [\[ISI\]](#) [\[Medline\]](#)

Anteby, E.Y., Hurwitz, A., Korach, O., Revel, A., Simon, A., Finci-Yeheskel, Z., Mayer, M. and Laufer, N. (1996) Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. *Hum. Reprod.*, **11**, 1947–1951. [\[Abstract\]](#)

Artini, P.G., Battaglia, C., D'Ambrogio, G., Barreca, A., Droghini, F., Volpe, A. and Genazzani, A.R. (1994) Relationship between human oocyte maturity, fertilization and follicular fluid growth factors. *Hum. Reprod.*, **9**, 902–906. [\[Abstract\]](#)

Balakier, H. and Stronell, R. (1994) Colour Doppler assessment of folliculogenesis in *in vitro* fertilization patients. *Fertil. Steril.*, **62**, 1211–1216. [\[ISI\]](#) [\[Medline\]](#)

Bassil, S., Magritte, J.P., Roth, J., Nissole, M., Donnez, J. and Gordts, S. (1995) Uterine vascularity during stimulation and its correlation with implantation in in-vitro fertilization. *Hum. Reprod.*, **10**, 1497–1501. [\[Abstract\]](#)

Bassil, S., Wyns, C., Toussaint-Demylle, D., Nissole, M., Gordts, S. and Donnez, J. (1997) The relationship between ovarian vascularity and the duration of stimulation in in-vitro fertilization. *Hum. Reprod.*, **12**, 1240–1245. [\[ISI\]](#) [\[Medline\]](#)

Battaglia, C., Larocca, E., Lanzani, A., Valentini, M. and Genazzani, A.R. (1990) Doppler ultrasound studies of the uterine arteries in spontaneous and IVF stimulated ovarian cycles. *Gynecol. Endocrinol.*, **4**, 245–250. [\[ISI\]](#) [\[Medline\]](#)

Battaglia, C., Artini, P.G., D'Ambrogio, G., Galli, P.A. and Genazzani, A.R. (1994) Uterine and ovarian blood flow measurement. Does the full bladder modify the flow resistance? *Acta Obstet. Gynecol. Scand.*, **73**, 716–718. [\[ISI\]](#) [\[Medline\]](#)

Battaglia, C., Artini, P.G., Giulini, S., Salvatori, M., Maxia, N., Petraglia, F. and Volpe, A. (1997) Colour Doppler changes and thromboxane production after ovarian stimulation with gonadotrophin-releasing hormone agonist. *Hum. Reprod.*, **12**, 2477–2482. [\[Abstract\]](#)

Battaglia, C., Salvatori, M., Maxia, N., Petraglia, F., Facchinetti, F. and Volpe, A. (1999) Adjuvant L-arginine treatment for *in vitro* fertilization in poor responders patients. *Hum. Reprod.*, **14**, 1690–1697. [\[Abstract/Free Full Text\]](#)

Ben-Shlomo, I., Adashi, E.Y. and Paync, D.W. (1994) The morphogenic/cytotoxic and prostaglandin stimulating activities of interleukin-1b in the rat ovary are NO independent. *J. Clin. Invest.*, **49**, 1463–1469.

Clancy, R.M. and Abramson, S.B. (1992) Novel synthesis of S-nitrosoglutathione and degradation by human neutrophils. *Anal. Biochem.*, **204**, 365–371. [\[ISI\]](#) [\[Medline\]](#)

Coulam, C.B., Bustillo, M. and Soenksen, D.M. (1994) Ultrasonographic predictors of implantation after assisted reproduction. *Fertil. Steril.*, **62**, 1004–1010. [\[ISI\]](#) [\[Medline\]](#)

Eldar-Geva, T., Lowe, P.J.M., MacLachlan, V., Rombauts, L. and Healy, D.L. (1998) Different influence of incongruent follicular development on *in vitro* fertilization–embryo transfer and gamete intrafallopian transfer pregnancy rates. *Fertil. Steril.*, **70**, 1039–1043. [\[ISI\]](#) [\[Medline\]](#)

Erikson, G.F., Garzo, V.G. and Margoffin, S.A. (1989) Insulin-like growth factor I regulates aromatase activity in human granulosa and granulosa luteal cells. *J. Clin. Endocrinol. Metab.*, **69**, 716–724. [\[Abstract\]](#)

- Facchinetti, F., De Martis, S., Neri, I., Caputo, A. and Volpe, A. (1997) Effects of transdermal glyceryltrinitrate on 24-h blood pressure changes in patients with gestational hypertension. *Prenat. Neonat. Med.*, **2**, 22–28.
- Facchinetti, F., Valensise, H., Neri, I., Menghini, S., Romanini, C. and Volpe, A. (1998) Reduction of serum citrulline levels in women at term toward the day of labor onset. *Acta Obstet. Gynecol. Scand.*, **77**, 174–177. [\[ISI\]](#) [\[Medline\]](#)
- Franco, J.G., Baruffi, R.L.R., Mauri, A.L., Petersen, C.G. and Oliveira, J.B.A. (1994) Ovarian synchrony factor: a new ultrasound parameter in the prognosis of follicular rupture. *Hum. Reprod.*, **9**, 1250–1252. [\[Abstract\]](#)
- Gaulden, M. (1992) Maternal age effect: the enigma of Down syndrome and other trisomic conditions. *Mutat. Res.*, **296**, 69–88. [\[ISI\]](#) [\[Medline\]](#)
- Gonen, Y. and Casper, R. (1990) Prediction of implantation by the sonographic appearance of the endometrium during controlled ovarian stimulation for in-vitro fertilization (IVF). *J. In Vitro Fertil. Embryo Transf.*, **7**, 146–152. [\[ISI\]](#) [\[Medline\]](#)
- Goswamy, R.K., Williams, G. and Steptoe, P.C. (1988) Decreased uterine perfusion a cause of infertility. *Hum. Reprod.*, **3**, 955–958. [\[Abstract\]](#)
- Hackeloer, B.J., Fleming, R., Robinson, H.P., Adam, A.H. and Coutts, J.R.T. (1979) Correlation of ultrasonic and endocrinologic assessment of human follicular development. *Am. J. Obstet. Gynecol.*, **135**, 122–128. [\[ISI\]](#) [\[Medline\]](#)
- Khalifa, E., Brzyski, R.G., Oehninger, S., Acosta, A.A. and Muasher, S.J. (1992) Sonographic appearance of the endometrium: the predictive value for the outcome of in-vitro fertilization in stimulated cycles. *Hum. Reprod.*, **7**, 677–680. [\[Abstract\]](#)
- Koning, H.E., Amselgruber, W. and Russe, I. (1989) La microcirculation dans les follicules et les corps jaunes d'ovaires de bovins-une Atude anatomique par corrosion. *Contraception-Fertilité-Sexualité*, **17**, 179–186.
- Lizzi, F.L. and Mortimer, A.J. (1989) Bioeffects considerations for the safety of diagnostic ultrasound. *J. Ultrasound Med.*, **7** (Suppl.), S1–S38. [\[ISI\]](#)
- Manau, D., Balash, J., Jimenez, W., Fabregues, F., Civico, S., Casamitjana, R., Creus, M. and Vanrell, S.A. (2000) Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. *Hum. Reprod.*, **15**, 1295–1299. [\[Abstract/Free Full Text\]](#)
- Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142. [\[ISI\]](#) [\[Medline\]](#)
- Noyes, N., Liu, H.C., Sultan, K., Schattman, G. and Rosenwaks, Z. (1995) Endometrial thickness appears to be a significant factor in embryo implantation in in-vitro fertilization. *Hum. Reprod.*, **10**, 919–922. [\[Abstract\]](#)
- Ramsay, B., De Belder, A., Campbell, S., Moncada, S. and Martin, J.F. (1994) A nitric oxide donor improves uterine artery diastolic blood flow in normal early pregnancy and in women at high risk of pre-eclampsia. *Eur. J. Clin. Invest.*, **24**, 76–78. [\[ISI\]](#) [\[Medline\]](#)

Ramsay, B., Johnson, M.R., Leone, A.M. and Steer, P.J. (1995) The effect of exogenous oestrogen on nitric oxide production in women: a placebo controlled crossover study. *Br. J. Obstet. Gynaecol.*, **102**, 417–419. [\[ISI\]](#) [\[Medline\]](#)

Steer, C.V., Campbell, S., Tan, S.L., Crayford, T., Mills, C., Mason, B.A. and Colins, W.P. (1992) The use of transvaginal color flow imaging after *in vitro* fertilization to identify optimum uterine conditions before embryo transfer. *Fertil. Steril.*, **57**, 372–376. [\[ISI\]](#) [\[Medline\]](#)

Tao, M., Kodama, H., Kagabu, S., Fukuda, J., Murata, M., Shimizu, Y., Hirano, H. and Tanaka, T. (1997) Possible contribution of follicular interleukin-1 $\beta$  to nitric oxide generation in human pre-ovulatory follicles. *Hum. Reprod.*, **12**, 2220–2225. [\[Abstract\]](#)

Taymor, M.L. (1996) The regulation of follicle growth: some clinical implications in reproductive endocrinology. *Fertil. Steril.*, **65**, 235–247. [\[ISI\]](#) [\[Medline\]](#)

Van Blerkom, J., Antczak, M. and Schrader R. (1997) The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular flow characteristics. *Hum. Reprod.*, **12**, 1047–1055. [\[ISI\]](#) [\[Medline\]](#)

Weiner, Z., Thaler, I., Levron, J., Lewit, N. and Itskovits-Eldor, J. (1993) Assessment of ovarian and uterine blood flow by transvaginal colour Doppler in ovarian-stimulated women: correlation with the number of follicles and steroid hormone levels. *Fertil. Steril.*, **59**, 743–749. [\[ISI\]](#) [\[Medline\]](#)

Yaron, Y., Botchan, A., Amit, A., Peyser, M.R., David, M.P. and Lessing, J.B. (1994) Endometrial receptivity in the light of modern assisted reproductive technologies. *Fertil. Steril.*, **62**, 225–232. [\[ISI\]](#) [\[Medline\]](#)

Zaidi, J., Pittrof, R. and Shaker, A. (1996) Assessment of uterine artery blood flow on the day of human chorionic gonadotropin administration by transvaginal color Doppler ultrasound in an *in vitro* fertilization program. *Fertil. Steril.*, **65**, 377–381. [\[ISI\]](#) [\[Medline\]](#)

Zaidi, J., Campbell, S., Pitroff, R. and Tan, S.L. (1995a) Endometrial thickness, morphology, vascular penetration and velocimetry in predicting implantation in an *in vitro* fertilization program. *Ultrasound Obstet. Gynecol.*, **6**, 191–198. [\[ISI\]](#) [\[Medline\]](#)

Zaidi, J., Jurkovic, D., Campbell, S., Okokon, E. and Tan, S.L. (1995b) Circadian variation in uterine artery blood flow indices during the follicular phase of the menstrual cycles. *Ultrasound Obstet. Gynecol.*, **5**, 406–410. [\[ISI\]](#) [\[Medline\]](#)